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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	Apr 95	Final - 10/1/90 - 9/31/93

4. TITLE AND SUBTITLE
Role of Proteogylcans in Cellular Signaling

5. FUNDING NUMBERS
DAAL03-90-G-0210

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8. PERFORMING ORGANIZATION REPORT NUMBER
N/A

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Research Office
P. O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER
ARO 28167.1-LS-SAH

11. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
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12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Proteoglycans have the ability to regulate the activity of several growth factors important to wound healing, including basic fibroblast growth factor and transforming growth factor-beta. In this project, we surveyed proteoglycan synthesis in a number of strains of normal human skin fibroblasts and in fibroblast strains derived from benign dermal tumors (keloids) which show a prolonged wound healing response and abnormal responses to hydrocortisone in tissue culture. Anion exchange chromatography was used to separate radiolabeled proteoglycans into heparan sulfate and dermatan sulfate charge classes. The most consistent and dramatic trend observed for all strains was the apparent hydrocortisone-induced decrease of dermatan sulfate proteoglycan accumulation in the cell-associated fraction from normal strains but not from keloid strains. Experiments are in progress to measure the messenger RNA for several dermatan sulfate proteoglycan core proteins known to be synthesized by human skin fibroblasts. Since the data collected so far are still incomplete, we intend to finish this project by measuring message levels for these core proteins mentioned above. Once these experiments are completed, we plan to publish these data and conclude this project.

14. SUBJECT TERMS
Proteoglycans, human skin fibroblasts, keloids, wound healing, hydrocortisone

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

NSN 7540-01-280-5500

Standard Form 298 (Rev 2-89)
Prescribed by ANSI Std 239-18
298-102

FINAL REPORT

A) ARO PROPOSAL NUMBER: 28167-LS-SAH

B) PERIOD COVERED BY REPORT: 10/1/90-9/31/93

C) TITLE OF PROPOSAL: Proteoglycans in Cellular Signaling

D) CONTRACT OR GRANT NUMBER: DALL03-90-0210

E) NAME OF INSTITUTION: Meharry Medical College

F) AUTHOR OF REPORT: Diane A. Blake, Ph.D.

G) LIST OF MANUSCRIPTS SUBMITTED OF PUBLISHED BY THIS PROJECT UNDER ARO SPONSORSHIP DURING THIS REPORTING PERIOD: 3 manuscripts in preparation which will be forwarded to ARO upon submission.

H) SCIENTIFIC PERSONNEL SUPPORTED: Diane A. Blake, Ph.D., Assistant Professor; Amitabha Chakrabarti, Ph.D., Postdoctoral Research Associate, Sandra Gamble, M.S., Research Assistant, Pamela Johnson, B.S., Graduate Student

Ms. Johnson, a graduate student supported by a Patricia Robert Harris Fellowship, is using a portion of this ARO-sponsored project to satisfy the requirements for the Ph.D. degree; although no degree was awarded during the tenure of this project, a copy of her thesis will be forwarded when it has been completed.

I) REPORT OF INVENTIONS: none

FINAL TECHNICAL REPORT

Low passage human skin fibroblasts of normal and keloid origin were obtained from Dr. Shirley Russell of Meharry Medical College and Dr. Patricia Hebda of the University of Pittsburgh. These low passage strains were metabolically labelled with ^{35}S-sulfate which incorporates predominantly (>95%) into the glycosaminoglycan chains of proteoglycans. The results from these experiments attempt to answer three questions: 1) is total proteoglycan accumulation altered in keloid versus normal cells, 2) can differences in the accumulation of heparin sulfate and chondroitin/dermatan sulfate proteoglycan be resolved between normal and keloid strains, 3) does hydrocortisone effect proteoglycan synthesis differently in keloid and normal strains. These results have been divided into three sections: I) total proteoglycan accumulation, II) heparan sulfate and dermatan/chondroitin sulfate proteoglycan accumulation, and III) effect of hydrocortisone treatment on proteoglycan accumulation in human skin fibroblasts derived from normal and keloid tissue.

I. Total proteoglycan accumulation

Purpose: To survey normal and keloid strains of low-passage human skin fibroblasts in order to determine if total proteoglycan accumulation is altered in keloid strains and how hydrocortisone effects total proteoglycan accumulation.

Procedure: A. Cell culture and ^{35}S-sulfate metabolic labelling

B. Harvest of secreted and cell-associated fractions

C. Paper chromatography to separate proteoglycans from unincorporated precursor

A. Cell culture and ^{35}S-sulfate metabolic labelling

Early passage normal and keloid stock cultures were obtained from Dr. Shirley Russell

of Meharry Medical College and Dr. Patricia Hebda at the University of Pittsburgh. Each strain was plated at 2.5 x 10^4 cell/ ml into eight 60 mm dishes for metabolic labelling and twenty 35 mm dishes for an accompanying growth curve. Cultures were maintained in a medium containing 10% fetal bovine serum and Ham's F-10 or 10% fetal bovine serum and Ham's F-10 plus 1.5 μM hydrocortisone (HC); medium was replaced daily from day 2 after plating. DNA per culture dish was assayed to generate a growth curve. The DNA assay employed a fluorescent Hoescht dye #33258. The growth curve was used to monitor the time at which the cells reached confluence; cells were labelled when they reached confluence. Strains entered this stage at day 7-9 after plating. Cultures were labelled for 18 hours in 2 ml of media containing 300 μCi/ml ^{35}S-sulfate.

B. Harvest of secreted and cell-associated fractions

The secreted and cell-associated fractions were harvested as described in Blake and McLean (1). Briefly, the radiolabeled culture medium was removed and the cell layer was washed twice with PBS; the media and washes were combined to form the **secreted** fraction. The **cell-associate** fraction was prepared by solubilizing the cell layer remaining in the culture dish in 4 M guanidine hydrochloride containing protease inhibitors. Both the secreted and cell-associated fraction were dialyzed into 8 M urea before analysis by paper chromatography and anion exchange HPLC.

C. Paper chromatography to separate proteoglycans from unincorporated precursor

Secreted and cell-associated samples containing at least 2000 cpm were spotted on Whatman 3 paper strips (N=4). The strips were placed in a resolving solvent containing butanol, acetic acid, and 1 N ammonium hydroxide in a ratio 4:6:3. Sulfated labelled proteoglycans remain at the origin while small species like free sulfate migrate down the strip; total ^{35}S-sulfate incorporation can be calculated from origin counts.

Results:

No dramatic increase or decrease in ^{35}S-sulfate incorporation were observed in the low passage keloid strains when compared to the low passage normal strains. Hydrocortisone also had little effect on ^{35}S-sulfate incorporation on three of the five strains studied, but a reduction of ^{35}S-sulfate incorporation was observed in one normal and one keloid strain. These data are presented in Figure 1.

II. Heparan sulfate and dermatan sulfate proteoglycan accumulation

Purpose: To survey several normal and keloid strain for differences in the accumulation of heparan sulfate and dermatan sulfate proteoglycans using anion exchange chromatography.

Procedure:

A Dionex BioLC HPLC equipped with a Dionex ProPac PA1 anion exchange column was used to resolve heparan sulfate from dermatan/chondroitin sulfate proteoglycans as described in Blake and McLean (1). Radiolabeled samples were eluted with a NaCl gradient during a 55 minute chromatogram, and proteoglycans labelled with ^{35}S-sulfate were detected using an on-line radioisotope detector.

Results:

Anion exchange HPLC was used to separate ^{35}S-sulfate labelled proteoglycans into dermatan sulfate and heparan sulfate charge classes. Previous studies with human skin fibroblasts (1) have shown that the material which elutes with a retention time of about 32 minutes is predominantly composed of heparan sulfate proteoglycans (HSPG) and the material in the second major peak (retention time = 37 min) is chondroitin/dermatan sulfate proteoglycans (CS/DSPG).

The anion exchange profiles of the secreted fractions from normal, keloid strains suggest that little HSPG (20% of total proteoglycan) accumulates in the secreted fraction.

The HS peak disappears in the secreted fractions from both normal and keloid cultures treated with hydrocortisone. A representative chromatogram of the secreted fraction from control and hydrocortisone treated cultures is shown in Figure 2.

The anion exchange chromatograms of the cell-associated fraction from all strains studied suggest that hydrocortisone either stimulates HSPG accumulation in this fraction or prevents its release into the secreted fraction. However, it seems unlikely that hydrocortisone simply prevents the release of HSPG into the secreted fraction because in two out of three strains tested , the increase in HSPG accumulation in the cell-associated fraction is two to three-fold greater than the decrease in HSPG accumulation in the secreted fraction. HSPG accumulation in strains treated with hydrocortisone ranged between 25-70% above control values . The anion exchange profiles of the cell-associated fractions suggest that hydrocortisone depresses CS/DSPG accumulation in most normal strains (See Figure 2A and 2B); hydrocortisone reduces the CS/DSPG peak from 50-73% of control (See Figure 2B).

The most consistent and dramatic trend observed for all strains was the apparent hydrocortisone-induced decrease of CS/DSPG accumulation in the cell-associated fraction from normal strains but not from keloid strains. Representative chromatographic profiles of the cell-associated fractions from norma and keloid strains as shown, respectively, in Figs. 3 and 4. Anion exchange chromatography of the cell-associated fractions from both low and later passage strains suggested that although hydrocortisone did not seem to substantially effect ^{35}S-sulfate accumulation into CS/DSPGs in keloid strains, hydrocortisone does reduce CS/DSPG accumulation in normal strains. These data are summarized in Table 1.

Since hydrocortisone appeared to depress the synthesis of cell-associated dermatan sulfate proteoglycans. We next intend to determine the message levels of DSPG core proteins to attempt to discover which DSPG core protein(s) may be downregulated. It is also possible that hydrocortisone may be decreasing the synthesis of glycosaminoglycan chains. However, an abbreviated review of glucocorticoids and proteoglycans literature suggest that glucocorticoids have a stimulatory effect on glycosaminoglycan synthesis (2-4). It has been reported that glucocorticoids stimulate ^{35}S-sulfate incorporation in several cell types including: human fetal lung tissue{(2), rabbit costal chondrocytes (3), and glomerular epithelial cells (4). Over the next 4-6 months we plan to determine if hydrocortisone is effecting the message

levels of the major skin CS/DSPG core, decorin. Hydrocortisone may also be affecting the expression of biglycan and versican, two other CS/DSPG cores commonly found in skin. I would like to determine the message levels for these cores respond to hydrocortisone; we have obtained a cDNA probe for the biglycan core protein from L. Fisher and can purchase cDNA probe for the versican core from Telios Pharmaceuticals. In addition to screening for these cores, we will determine message levels for the chondroitin sulfate/heparan sulfate proteoglycan, betaglycan (Transforming growth factor β (TGF-β) type III receptor). We are particularly interested in betaglycan because it mediates TGF-β interaction with its signalling receptor. Betaglycan is a cell-associated proteoglycan, and it is the cell-associated proteoglycans which appear to be most altered by hydrocortisone. We plan to write J. Massagué for a cDNA probe for the betaglycan core protein. Since we have probes for the HSPG cores, glypican and perlecan, we will also resolve how message levels of these cores are effected by hydrocortisone. This would substantiate preliminary results started by a former post-doctoral fellow (Dr. Chakrabarti) in the laboratory.

Since the data collected so far are still incomplete, we intend to finish this project by measuring message levels for the various proteoglycan core proteins mentioned above. Once these experiments are completed, we plan to publish these data and conclude this project.

References

1. Blake, D.A. and McLean, N.V. (1990) *Anal. Biochem.* **190:** 158-164

2. Heifetz, A. and Snyder, J.M. (1981) *J. Biol. Chem.* **256:** 4957-4967.

3. Kato, Y. and Gospodarowicz, D. (1985) *J. Biol. Chem.* **260:** 2364-2373.

4. Kasinath, B.S., Singh,A.K., Kanwar,Y.S., and Lewis, E.J. (1990) *J. Lab. Clin. Med.* **115:** 196-202.

Table 1. Effect of hydrocortisone on the incorporation of ^{35}S-sulfate into cell-associated HSPG and DSPG of human skin fibroblasts

	HSPG		DSPG	
STRAIN	Untreated	HC treated	Untreated	HC treated
	cpm/μg DNA			
Normal				
21	2212± 202*#	1125± 98	4843± 388*#	1651± 127
131	3674± 144*	3124± 204	9570± 281*#	3527± 141
103N5	2107± 32	3046± 42	2743± 32	2883± 384
132	3627± 280	2321±414	4098± 130*#	1496± 59
116	3458± 206*#	1915± 29	4291± 100*#	1411± 71
108	2466± 45	2345± 34	2741± 60*#	1384± 59
AlKo	2201± 176	2311± 323	4674± 375*#	1527± 183
EdYo	4366± 413	5509± 389	3765± 48	4623± 91
HaZi	2095± 313	2784± 405	2681± 317	2259± 276
RoCo	2453± 429	3922± 180	7547± 307*	5316± 137
Keloid				
33	1824± 143	1948± 212	2492± 349	2311± 399
125	1946± 347	2043± 264	2759± 96	2409± 135
124	1983± 197	1726± 90	3174± 123*	2309± 124
30	2017± 40	2214± 69	2885± 292	2418± 124
MoMo	2871± 1247	2016± 50	4690± 205*	3175± 120

and # indicate a significant decrease in ^{35}S-sulfate incorporation after treatment with hydrocortisone (* - 95% confidence interval; # - 99% confidence interval)

Figure Legends

Figure 1. The effect of hydrocortisone on total ^{35}S-sulfate incorporation in human skin fibroblasts of normal and keloid origin. Confluent cultures were radiolabeled and analyzed as described on page 3 of this report.

Figure 2. Anion exchange chromatography of secreted proteoglycans from normal human skin fibroblasts: The effect of hydrocortisone. Panel A, radiolabeled sample derived from the secreted fraction of the normal strain AlKo; Panel B, radiolabeled sample derived from the secreted fraction of the same strain, AlKo, cultured in the presence of hydrocortisone. Note the disappearance of the peak eluting at 35 minutes in the hydrocortisone-treated sample.

Figure 3. Anion exchange of cell-associated proteoglycans from normal human skin fibroblasts: The effect of hydrocortisone. Panel A, radiolabeled sample derived from the cell-associated fraction of the normal strain, AlKo; Panel B, radiolabeled sample derived from the cell-associated fraction of the same strain, AlKo, cultured in the presence of hydrocortisone. Note the disappearance of the peak eluting between 35 and 40 minutes in the hydrocortisone-treated sample.

Figure 4. Anion exchange chromatography of cell-associated proteoglycans from keloid human skin fibroblasts: Hydrocortisone has no effect. Panel A, radiolabeled sample derived from the cell-associated fraction of the keloid strain, MoMo; Panel B, radiolabeled sample derived from the same strain,MoMo, cultured in the presence of hydrocortisone. Note that hydrocortisone has little effect on the chromatographic profile in the keloid strain..

Figure 1. The effect of hydrocortisone on total ^{35}S-sulfate incorporation

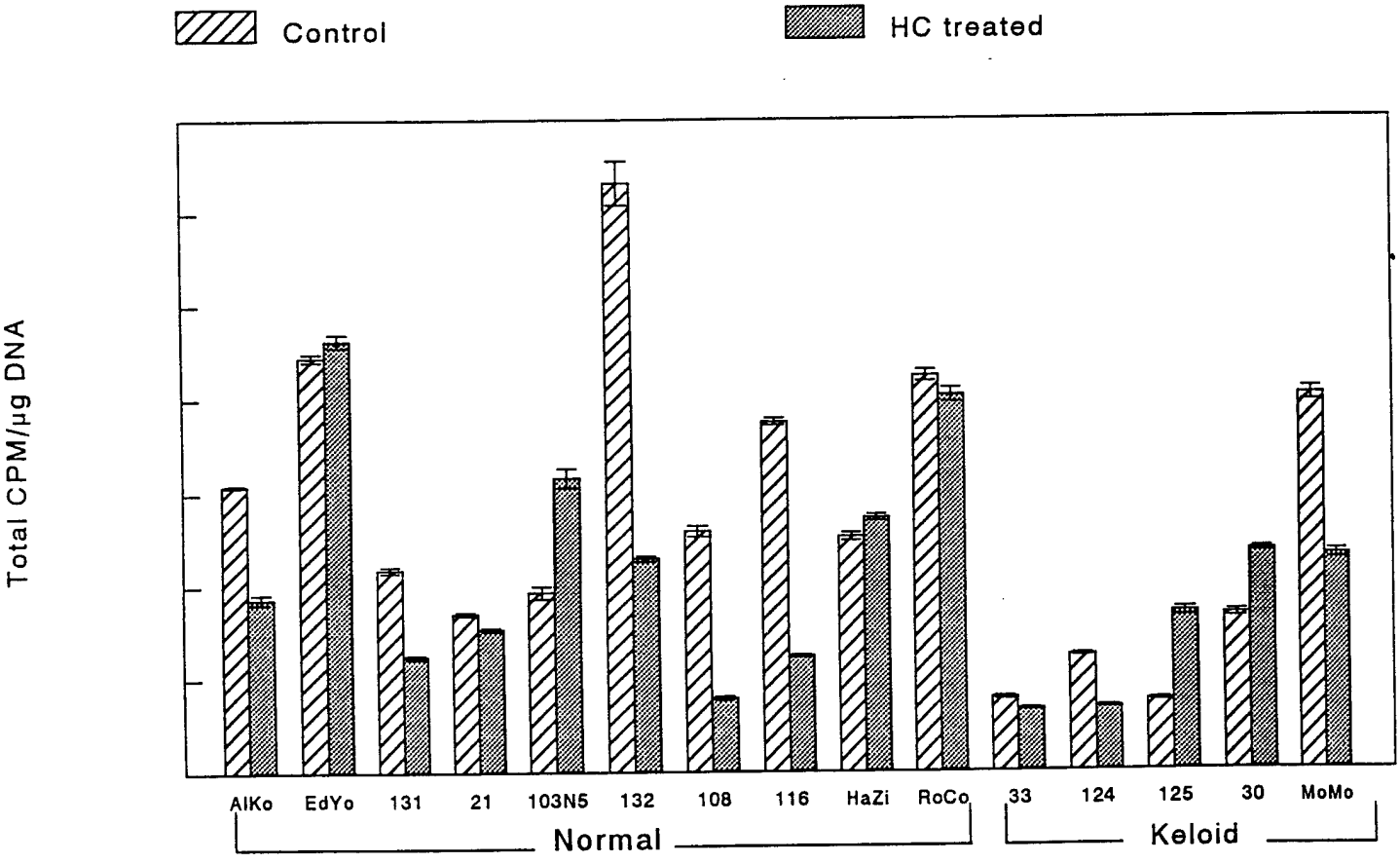


Figure 2. Anion exchange chromatography of secreted PGs from normal HSF

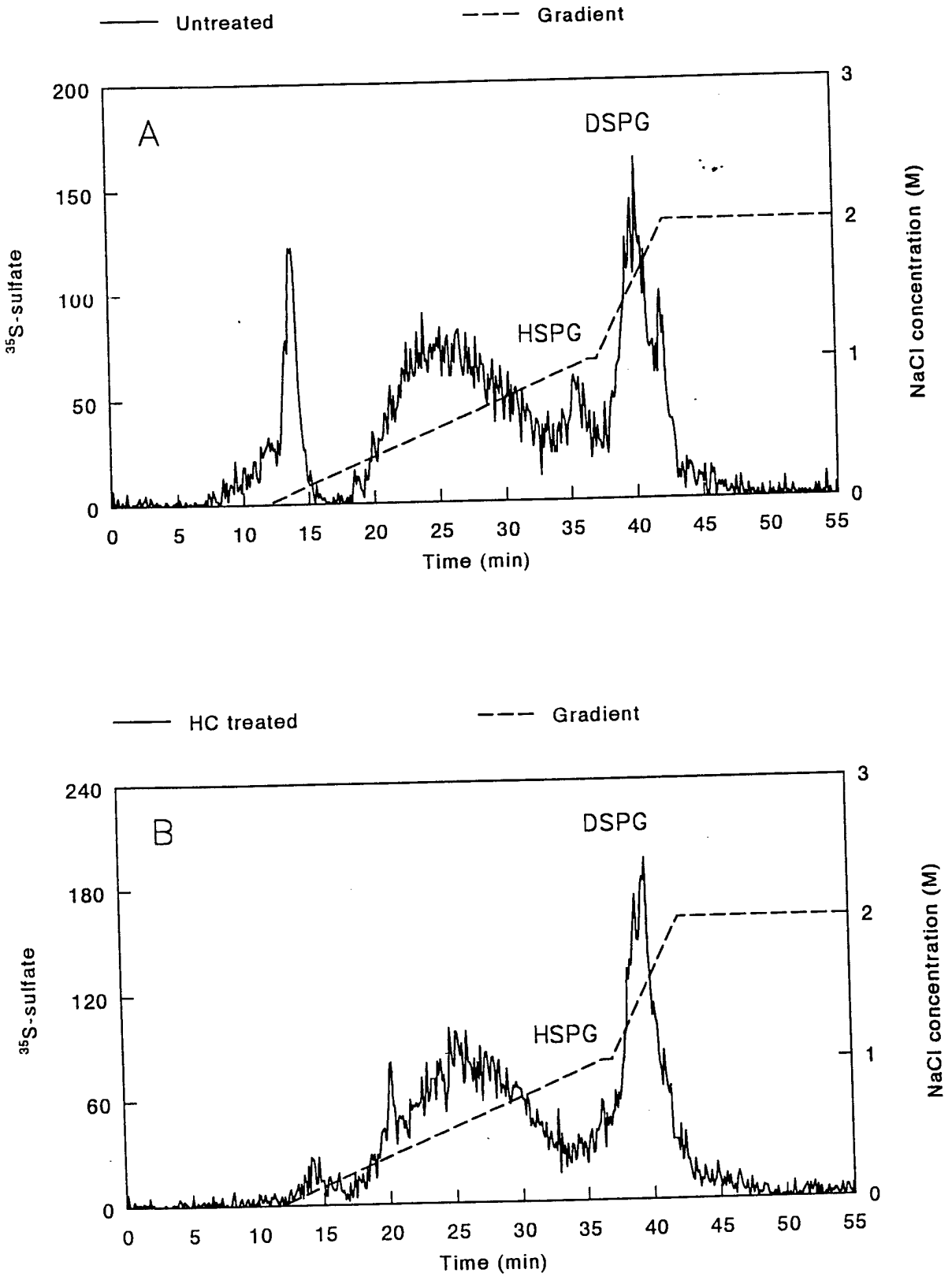


Figure 3. Anion exchange chromatography of cell-associated PGs from normal HSF

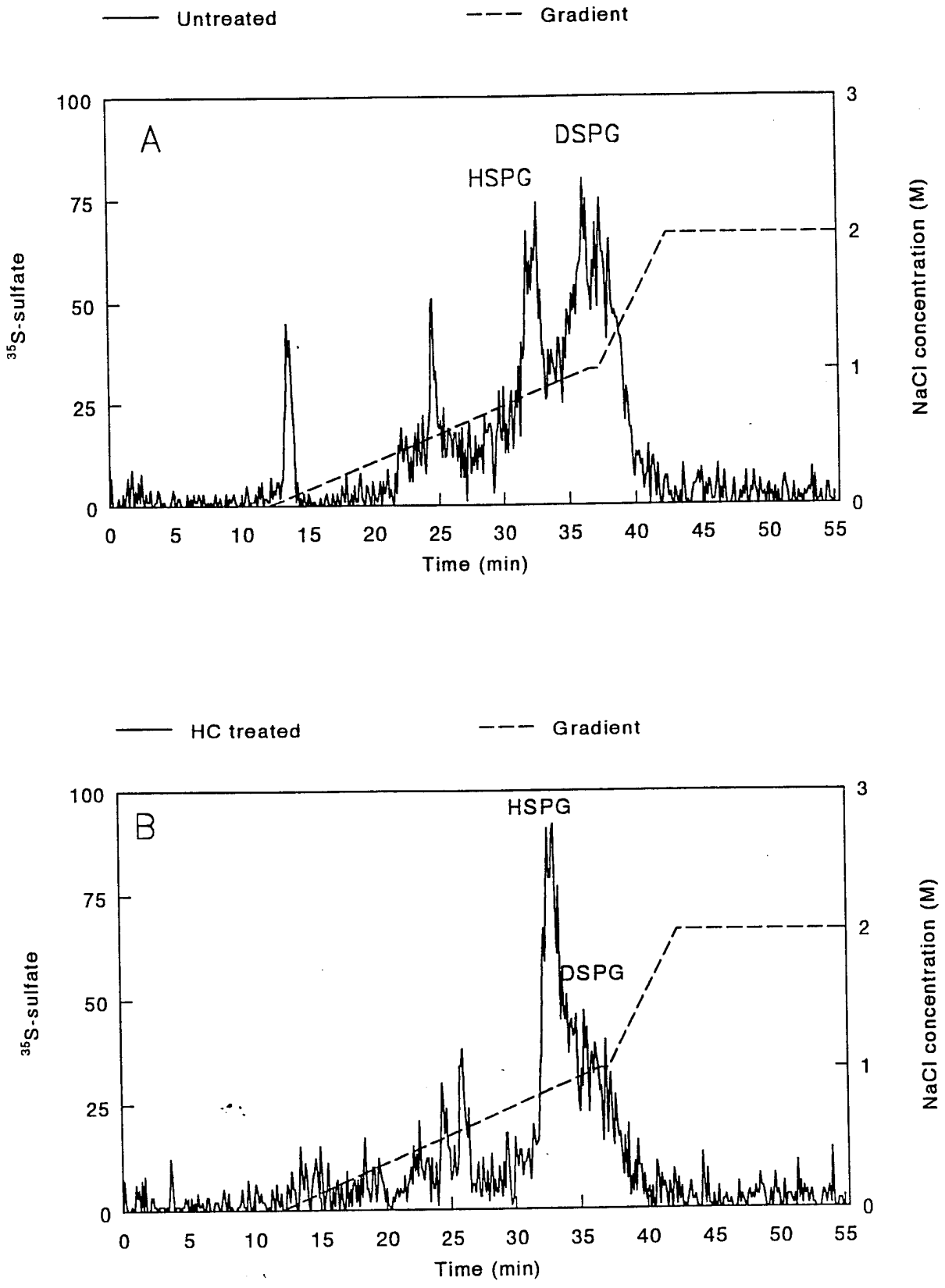
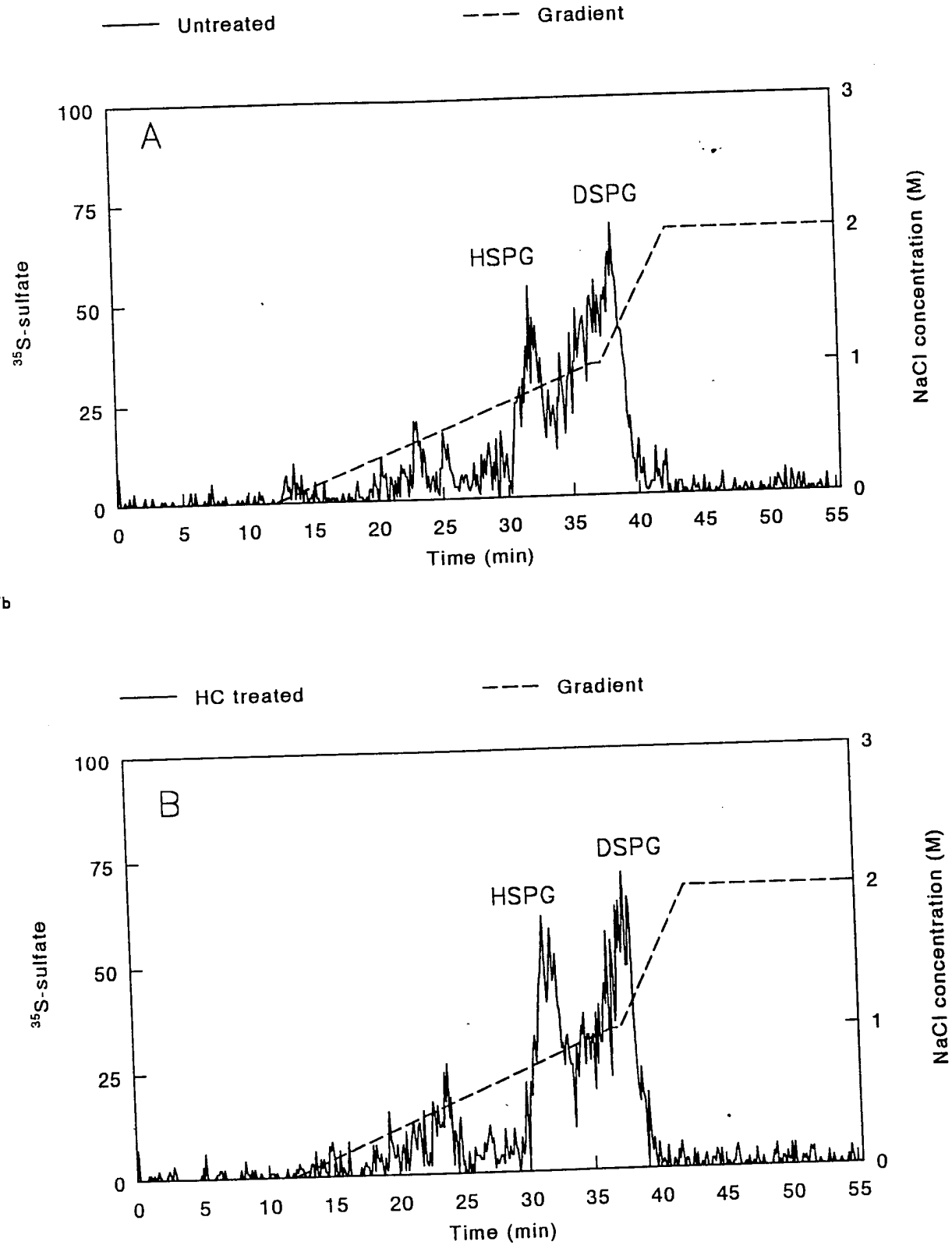


Figure 4. Anion exchange chromatography of cell-associated PGs from keloid HSF



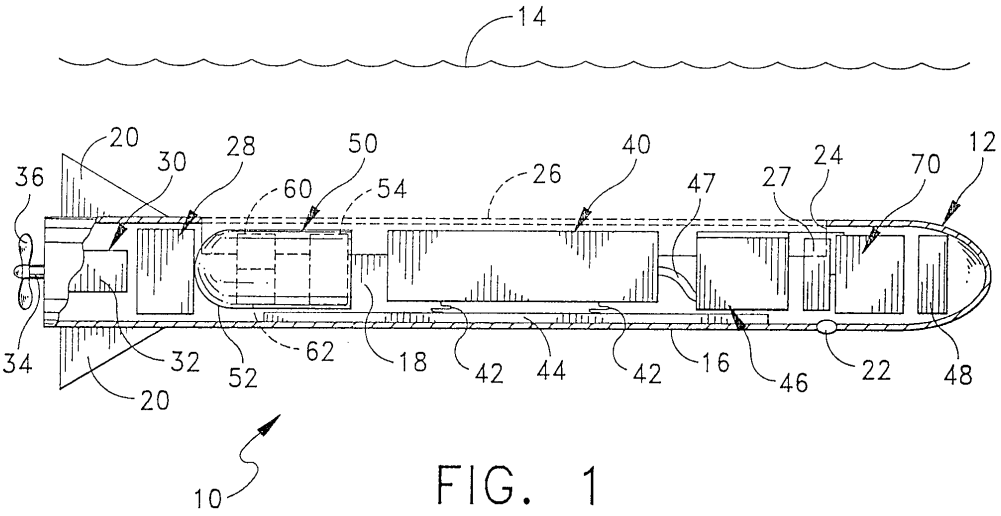

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FIG. 1

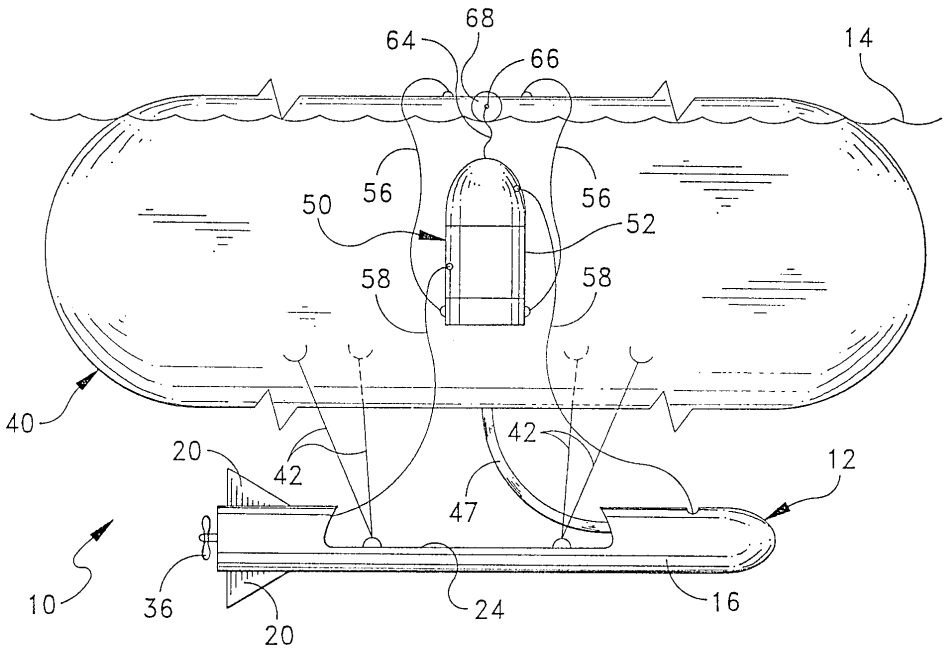
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FIG. 2

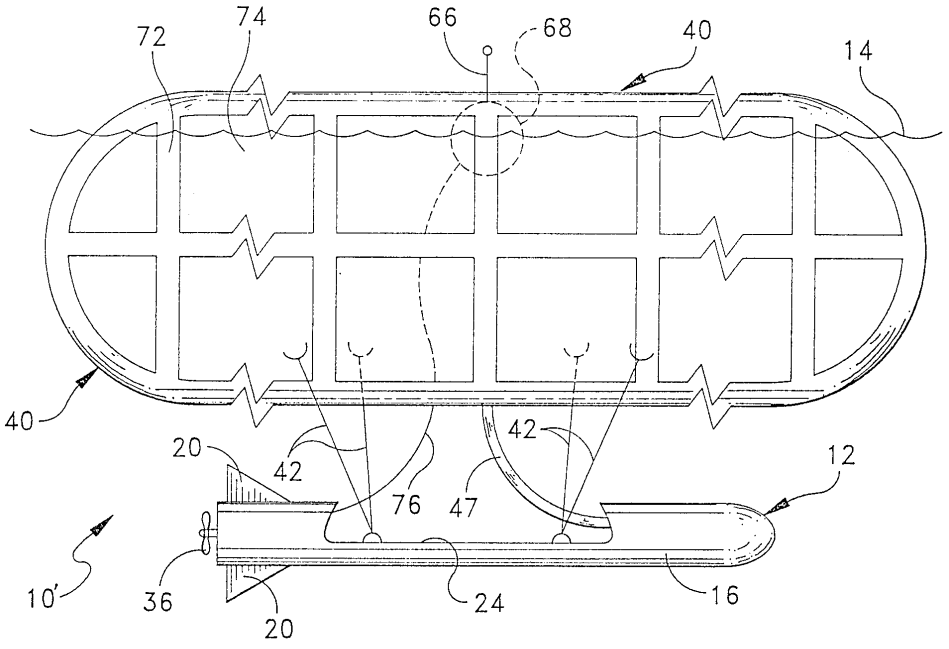

FIG. 3